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Biochemistry

Use of site-directed mutagenesis to identify an upstream regulatory sequence of *sodA* gene of *Escherichia coli* K-12

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ABSTRACT **Mn-containing superoxide dismutase (SodA; superoxide:superoxide oxidoreductase, EC 1.15.1.1) biosynthesis in *Escherichia coli* is regulated by several environmental stimuli. The DNA sequence of *sodA* shows the presence of a potential binding site for a regulatory protein(s) at the −35 region. To explore the possible role of this region in the regulation of *sodA*, we used oligonucleotide-directed site-specific mutagenesis to change the sequence of nucleotides −48 through −44 from 5′-GGCAT-3′ to 5′-TTACG-3′. We studied the effect of this altered sequence on the expression of *sodA*. The data showed that the altered sequence resulted in the constitutive expression of the gene. Thus, *E. coli* harboring a plasmid containing the mutated *sodA* gene (pSNM6) were uninducible by paraquat in aerobiosis or by 2,2′-dipyridyl in aerobiosis or anaerobiosis. Furthermore, a multicopy plasmid containing the mutated *sodA* failed to titrate the repressor molecules present in an *E. coli* strain carrying the *sodA–lacZ* fusion. In contrast, multicopy plasmids containing the wild-type *sodA* gene were able to titrate the repressor protein and to cause the anaerobic induction of β-galactosidase in this *sodA–lacZ* fusion strain. These results indicate that the region within and around the mutated sequence probably plays an important role in *sodA* regulation and that the mutation disrupts a sequence that interacts with the repressor.**

Superoxide dismutases (superoxide:superoxide oxidoreductase, EC 1.15.1.1) are ubiquitous among aerobic and aerotolerant organisms. They constitute an essential component of the biological defenses against oxygen toxicity (1–4). *Escherichia coli* possesses three isozymic forms of superoxide dismutase (5): a manganese-containing enzyme (SodA; ref. 6), an iron-containing enzyme (7), and a hybrid enzyme containing both manganese and iron (8, 9).

The regulation of SodA biosynthesis in *E. coli* has been extensively studied both *in vivo* and *in vitro* (9–21). Thus, the enzyme is induced by: oxygen (5, 10), redox-active compounds capable of generating superoxide radical in the presence of oxygen (11, 12), ferrous iron chelators under aerobiosis or anaerobiosis (13, 14), nitrate under anaerobiosis (9, 15, 16), and several strong oxidants capable of positively changing the redox potential of the cells (17). These findings led us to propose that the synthesis of SodA in *E. coli* is negatively regulated by an iron-containing trans-acting repressor protein (RPFe) (9, 13). It is also proposed that the redox state of the cells dictates the valence of the iron in RPFe (i.e., $RPFe^{2+}/RPFe^{3+}$), which in-turn regulates the synthesis of SodA (9, 13, 17). $RPFe^{2+}$ is presumed to be the active repressor, and the induction of the enzyme by oxygen, nitrate, redox-cycling compounds, and various oxidants is due to the oxidation of $RPFe^{2+}$ to $RPFe^{3+}$. Recent *in vivo* and *in vitro* findings (9, 17, 19–21) support this negatively controlled transcriptional model.

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The gene coding for SodA (*sodA*) has been cloned (22, 23) and sequenced (23). The sequence shows the presence of an almost perfect 19-base pair (bp) palindrome at the −35 region, which represents a potential binding site for a regulatory protein (23). A core consensus sequence within this palindrome shows some but not extensive homology to catabolite–gene activator protein (CAP) binding sites (23, 24). Also, possible integration-host-factor (IHF) binding sites have been identified within and around this palindrome (23, 25). IHF has been implicated as having a regulatory role in gene expression (26). To explore the possible role of this region in the regulation of *sodA*, we used oligonucleotide-directed site-specific mutagenesis to change nucleotides −48 through −44 overlapping the IHF binding site and the 19-bp palindrome, including the 5′ end of one of the two inverted repeats of the dyad region. The data presented in this report indicate that the region in and around the 19-bp palindrome plays an important role in *sodA* regulation.

MATERIALS AND METHODS

Materials. Methyl viologen (paraquat), 2,2′-dipyridyl, xanthine, xanthine oxidase, horse heart cytochrome *c*, RNase A, tetracycline, puromycin, kanamycin, ampicillin, chloramphenicol, dextran sulphate, Tris, and *o*-nitrophenyl β-D-galactoside were purchased from Sigma. Urea and phage T4 DNA ligase were obtained from BRL. Restriction enzymes and the Klenow fragment of DNA polymerase were from BRL, New England Biolabs, or Stratagene. T4 polynucleotide kinase was purchased from Promega. Protein encoded by gene *32* and dNTPs were from Pharmacia. The sequencing kit was obtained from Stratagene. [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) and adenosine 5′-[α-(^{35}S)-thio]triphosphate (500 Ci/mmol) were purchased from New England Nuclear. NEF-978 colony/plaque screens were from DuPont/NEN research products. The 28-mer oligonucleotide used in site-directed mutagenesis, 3′-TTTTCATGAATCATGAATCACTATTAGTAAAAGT-5′, was synthesized and purified by the Molecular Biology Center, Genetics Department, at North Carolina State University.

Bacterial Strains and Plasmids. *E. coli* XLI-Blue (Stratagene) strain *endA1*, *hsdR17* (r_K^-, m_K^+), *supE44*, *thi-1*, λ^-, *recA1*, *gyrA96*, *relA1*, Δ(*lac*), [F′,*ProAB*, *lacI*^{q}*Z*, Δ*M15*, Tn*10* (tetracycline resistance)]; QC779, a mutant *E. coli* strain (*sodA sodB*) (27); and QC772 (27), a *sodA–lacZ* fusion strain, were used throughout this study. pDT1-5 (22) was kindly provided by D. Touati (University of Paris VII). Bluescript SK(−) plasmid and VCSM13 phage were obtained from Stratagene.

Construction of pSN(−)4. *sodA* gene was isolated by digesting pDT1-5 with *Ava* I and purifying the *sodA* (1.05 kb)

Abbreviations: SodA, Mn-containing superoxide dismutase; IHF, integration-host-factor; LB broth, Luria–Bertani broth; Fur, ferric uptake-regulation protein.

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gene on N-45 DEAE membrane. The gene was cloned into Bluescript SK(−) plasmid at the *Xho* I–*Sma* I site by digesting the vector with *Ava* I (Fig. 1). The orientation of the gene in plasmid pSN(−)4 and the sequence of the 5′ untranslated region was determined by dideoxy sequencing (28). Plasmid DNA was isolated by the method of Birnboim and Doly (29).

Construction of pSNM6. Oligonucleotide-directed site-specific mutagenesis was carried out as described by Zoller and Smith (30) as modified in the Stratagene protocol. Fifty nanograms of the synthetic 28-mer oligonucleotide was phosphorylated by mixing with 2 μl of 10× kinase buffer (in Stratagene protocol), 2 μl of 10 mM ATP, and 4 units of T4 polynucleotide kinase in a 20-μl reaction mixture and was incubated at 37°C for 1 hr. The phosphorylated primer was annealed to 1 μg of single-stranded pSN(−)4 template DNA by incubating at 65°C for 10 min and was allowed to cool to room temperature for 5 min. Primer extension and ligation was carried out in a final volume of 40 μl by adding 2 μl of each dNTP at 2.5 mM, 4 μl of 10 mM ATP, 4 μl of 10× ligation buffer (in Stratagene protocol), 4 μg of gene *32* protein, 1.5 units of Klenow fragment, and 4 units of T4 DNA ligase to the annealing mixture. This synthesis reaction was carried out at room temperature for 4 hr. Competent XL1-B cells were transformed with 10 μl of the synthesis reaction mixture containing the heteroduplex DNA and were plated on Luria–Bertani (LB) broth (pH 7.3), containing ampicillin at 500 μg/ml. Colonies were transferred in duplicate onto NEF-978 colony screen filters, and duplicate filters were made. The 28-mer synthetic oligonucleotide was 5′-end-labeled by using 100 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) and was used for hybridization and identification of the colonies that contained a mixture of homoduplex mutant and parent pSN(−)4 plasmids. Colony hybridization was carried out as described (31, 32). The colonies containing homoduplex mutant plasmid (pSNM6) were recovered by a second screen of XLI-Blue cells transformed with DNA isolated from a positive colony from the first screen. DNA from these putative homogeneous mutant colonies was sequenced to confirm the site-directed mutation.

Media and Growth Conditions. *E. coli* cultures were grown in LB broth (pH 7.3) containing per liter 10 g of Bactotryptone (Difco), 5 g of yeast extract (Baltimore Biological Laboratory), and 10 g of NaCl. Media were supplemented with sterile $MnCl_2$ to a final concentration of 1 mM to provide sufficient Mn cofactor needed under highly induced conditions. Under anaerobic growth conditions, 0.5% glucose was added to LB broth to provide a fermentable carbon source. Ampicillin (500 μg/ml), chloramphenicol (20 μg/ml), tetracycline (10 μg/ml), kanamycin (70 μg/ml), and puromycin (150 μg/ml) were used as needed. Aerobic and anaerobic growth conditions were essentially the same as described (9, 13, 17).

Assays. Cells were harvested at 4°C by centrifugation, and cell-free extracts were prepared as described (9). Protein was

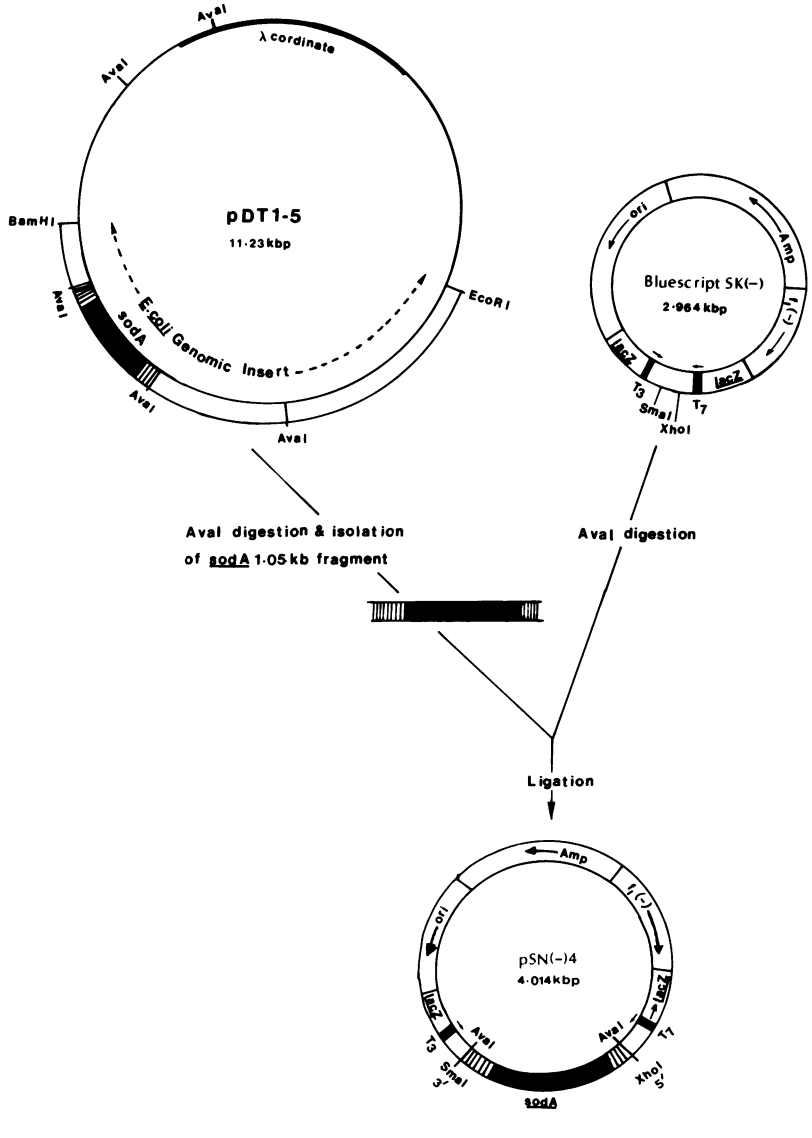

FIG. 1. Construction of pSN(−)4. *sodA* was isolated by digesting pDT1-5 with *Ava* I and was purified as described. The gene was cloned in Bluescript SK(−) plasmid at the *Xho* I–*Sma* I site by digesting the vector with *Ava* I.

assayed by the method of Lowry *et al.* (33). Superoxide dismutase was assayed by the cytochrome *c* method (1). Superoxide dismutase activity was visualized by using the nitro blue tetrazolium method (34) after separation of the proteins by electrophoresis on 10% polyacrylamide gels (35). β-Lactamase was assayed as described (36). β-Galactosidase was assayed as described by Miller (37). Proteins were separated by electrophoresis on sodium dodecyl sulfate (SDS)/10% polyacrylamide gels (38) and immunoblotted (39). Antiserum prepared against pure SodA was a gift of D. Clare (Department of Animal Science, North Carolina State University). The peroxidase conjugate of goat anti-rabbit immunoglobulin was purchased from Cappel/Cooper Biomedical. SodA bands were visualized indirectly by staining for peroxidase activity (40).

RESULTS

Sequence of the 5'-Untranslated Region of *sodA* from pSN(−)4 and pSNM6. It was imperative that the *sodA* gene subcloned form pDT1-5 into pSN(−)4 have the identical 5' untranslated sequence as that published for *sodA* from pSOD-1 (23). Indeed, this was the case. Fig. 2 shows a partial sequence of *sodA* from pSN(−)4 from nucleotide −59 through nucleotide +1, involving the IHF binding site and the 19-bp palindrome. Oligonucleotide-directed site-specific mutagenesis was used to alter the sequence of nucleotides −48 through −44 as described. DNA isolated from colonies containing the homoduplex mutant plasmid pSNM6 was sequenced to confirm the desired mutation in nucleotides −48 through −44 from 5'-GGCAT-3' to 5'-TTACG-3' (Fig. 2).

Effects of Paraquat and 2,2'-Dipyridyl on the Aerobic Synthesis of SodA from pSNM6. In aerobically grown wild-type *E. coli*, the synthesis of active SodA is induced by the presence of ferrous iron chelators (13, 14) or by redox-active compounds capable of exacerbating the intracellular flux of superoxide radicals (11, 12). The inductive effects of these compounds were shown to be at the transcriptional level (9, 12, 13, 19).

In this study, we compared the expression of SodA from the wild-type gene cloned in plasmid pSN(−)4 and from the mutant gene present in plasmid pSNM6. We reasoned that, if the mutated region of pSNM6 were indeed in or around the proposed (9, 13, 23) cis-acting regulatory site, then the expression of SodA from the mutant gene would be different from that of the wild-type gene.

We used *E. coli* strain QC779 (27), which lacks both SodA and the iron-containing superoxide dismutase, as a host for the plasmids carrying the wild-type or the mutant *sodA* gene. Thus, in this null background, the activity of SodA reflects the enzyme expressed from the plasmid introduced into the host strain. Plasmid pDT1-5, which carries wild-type *sodA* and 3.75 kilobases (kb) of adjacent chromosomal genes from *E. coli* K-12, was included for comparison. The effects of paraquat (0.1 mM) and 2.2'-dipyridyl (1 mM) on the aerobic expression of SodA by *E. coli* QC779 transformed with pDT1-5, pSN(−)4, or pSNM6 are shown in Table 1.

Table 1. Aerobic expression of SodA from *E. coli* (QC779) harboring plasmid pDT1-5, pSN(−)4, or pSNM6 in response to added paraquat or 2,2'-dipyridyl

Strain (plasmid)	SodA, units/mg		
	LB	LB + PQ	LB + 2,2'-dipyridyl
QC779	0	0	0
QC779 (pDT1-5)	96	458	256
QC779 [pSN(−)4]	362	1733	1143
QC779 (pSNM6)	715	739	723

Overnight cultures grown in LB broth (LB) containing 1 mM $MnCl_2$ plus the appropriate antibiotics (chloramphenicol, 20 μg/ml; ampicillin, 500 μg/ml) were used to inoculate fresh prewarmed medium of the same composition to an initial OD_{600} = 0.05. Paraquat (PQ) (0.1 mM) or 2,2'-dipyridyl (1.0 mM) were added when the OD_{600} of the cultures reached 0.1. Cultures were allowed to grow to an OD_{600} = 1.0 before cells were harvested and cell-free extracts were prepared and assayed for SodA, which is expressed in units per mg of soluble cellular proteins.

The data in Table 1 clearly indicate that *sodA* in pDT1-5 and pSN(−)4 was inducible by paraquat and by 2,2'-dipyridyl as expected (9, 13, 19), whereas the mutant *sodA* in pSNM6 was not inducible by either of these known inducing agents. In all cases, boiling the samples for 5 min resulted in complete loss of SodA activity, thus ensuring the absence of free (i.e., unchelated) Mn^{2+} in the dialyzed samples, which is known to interfere with the assay for superoxide dismutase (41). The expression of SodA, in the absence of added inducing agents, was much higher from plasmid pSNM6 than from either pSN(−)4 or pDT1-5. It is also interesting to note that the expression of SodA from plasmid pSN(−)4 was consistently about 4-fold higher than that expressed from plasmid pDT1-5, possibly related to differences in their copy number. Previous studies have shown that the level of ampicillin resistance (expressed as β-lactamase, the product of *bla* gene) is directly proportional to the copy number of the gene (42). Therefore, we determined the specific activity of β-lactamase in QC779 harboring each of the plasmids and found that β-lactamase was expressed 2.5 ± 0.2-fold higher from cells harboring pSN(−)4 or pSNM6 than from cells harboring pDT1-5 (L. W. Schrum and H .M.H., unpublished data). These relative copy numbers for plasmids pSN(−)4/pDT1-5 as depicted from the level of β-lactamase expression agreed well with the relative copy numbers reported for pUC/pBR322-derived plasmids (43).

Anaerobic Expression of SodA from pSNM6: Effect of 2,2'-Dipyridyl. Previous studies have shown that *sodA* gene is repressed under normal anaerobic growth (5, 9, 13, 17, 19). Immunoblot (Western blot) analysis of cell-free extracts prepared from anaerobically grown cells demonstrated the absence of SodA antigen (17, 27). Ferrous iron chelators and strong oxidants have been shown to induce the synthesis of SodA under anaerobic conditions (9, 13, 14, 17).

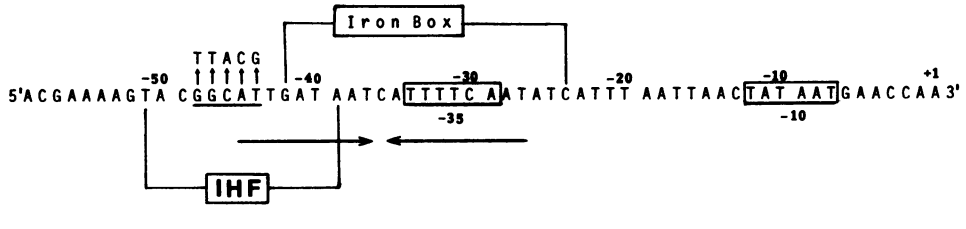

FIG. 2. Nucleotide sequence of the 5' region of *sodA*. Only nucleotides −59 to +1 (23) are listed. The +1 nucleotide designates the start point of transcription. The −35 and −10 regions are boxed. Arrows indicate the 19-bp palindrome. The IHF- and Fur (iron box)-binding sites are bracketed. Underlined nucleotides (−48 through −44) indicate the nucleotides changed in plasmid pSNM6 by site-specific mutagenesis.

Table 2. Anaerobic expression of SodA

Strain (plasmid)	SodA, units/mg	
	LB	LB + 2,2'-dipyridyl
QC779	0	0
QC779 (pDT1-5)	7.0	23.0
QC779 [pSN(−)4]	112.0	212.0
QC779 (pSNM6)	156.0	186.0

Anaerobic LB broth (LB) supplemented with glucose (0.5%), $MnCl_2$ (1 mM), and chloramphenicol (20 μg/ml) was used for growth. Ampicillin (500 μg/ml) was added to cultures transformed with the different plasmids. Overnight cultures (15–17 hr) were used at 1% to inoculate fresh prewarmed medium of the same composition. 2,2'-Dipyridyl (1 mM) was added when the OD_{600} reached 0.1. Cultures were harvested in late logarithmic phase of growth (6–7 hr). Puromycin (150 μg/ml) was added before the flasks were stoppered and removed from the anaerobic chamber. Cells were harvested, and cell-free extracts were prepared and assayed.

In the present study, *E. coli* QC779 transformed with pDT1-5, pSN(−)4, or pSNM6 were grown anaerobically and tested for the expression of SodA in response to the presence or absence of 2,2'-dipyridyl (Table 2). The data clearly show that the presence of multicopy plasmids harboring a wild-type *sodA* gene [i.e., pDT1-5 and pSN(−)4] resulted in the anaerobic expression of SodA in the absence of 2,2'-dipyridyl. The presence of pDT1-5 in the cells resulted in the synthesis of active SodA (7 units/mg of protein), whereas the presence of pSN(−)4 resulted in a further 16-fold increase in the expression of the enzyme. The anaerobic expression of *sodA* in response to the presence of these multicopy plasmids, pDT1-5 and pSN(−)4, that harbor the wild-type gene is presumably due to their capacity to neutralize the limited number of repressor molecules normally present in the cell (9, 19). The data also indicate that pSN(−)4 is more efficient than pDT1-5 in neutralizing the repressor molecules, probably because of its higher copy number per cell. Furthermore, the addition of 2,2'-dipyridyl to cells harboring pDT1-5 or pSN(−)4 resulted in a further 2- to 3-fold increase in the anaerobic expression of active SodA (Table 2). On the other hand, cells harboring the mutant plasmid pSNM6 showed a higher level of expression of SodA that was not significantly affected by the presence of 2,2'-dipyridyl (Table 2). Data in Fig. 3 show that the amount of active SodA expressed by cells harboring plasmids pDT1-5, pSN(−)4, or pSNM6 correlates with the amount of SodA antigen present in the cells.

Plasmid pSNM6 Does Not Neutralize the *sodA* Repressor. The above results suggest that the mutational changes introduced in plasmid pSNM6 must have altered the cis-acting regulatory site of *sodA* in a fashion that rendered the gene to be constitutively expressed under aerobic and anaerobic growth conditions. To strengthen this conclusion, we designed an experiment to demonstrate that the anaerobic expression of SodA from plasmid pSNM6, unlike pDT1-5 or pSN(−)4, is not due to the neutralization of the repressor molecules. We used an isogenic strain of *E. coli* that contains a chromosomal *sodA–lacZ* fusion (27). In this strain, the expression of β-galactosidase is under the control of the *sodA* promoter, and induction of β-galactosidase faithfully mimics that of SodA (19). Thus, in this strain (QC772), β-galactosidase is not expressed in anaerobiosis; and the presence of plasmid pDT1-5 results in low expression of β-galactosidase (19).

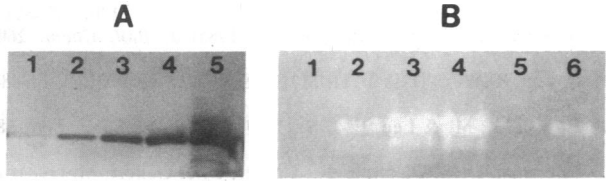

FIG. 3. Anaerobic expression of SodA. (*A*) Western blot analysis of anaerobic cell-free extracts (15 μg of protein per lane) after electrophoresis on SDS/10% polyacrylamide gel. Lanes: 1, QC779; 2, QC779 (pDT1-5); 3, QC779 [pSN(−)4]; 4, QC779 (pSNM6); 5, pure *E. coli* SodA. (*B*) SodA activity stain of anaerobic cell-free extracts (100 μg of protein in lanes 1–4 and 10 μg of protein in lanes 5 and 6). Lanes: 1, QC779; 2, QC779 (pDT1-5); 3 and 5, QC779 [pSN(−)4]; 4 and 6, QC779 (pSNM6).

Table 3. Effect of pSNM6 on the anaerobic expression of β-galactosidase (β-Gal) in the *sodA–lacZ* fusion strain

Strain (plasmid)	OD_{600} at 2 hr	β-Gal, units per OD_{600}
QC772	0.27	20.0
QC772 (pDT1-5)	0.48	26.0
QC772 [Sk(−)]	0.47	11.0
QC772 [pSN(−)4]	0.33	173.0
QC772 (pSNM6)	0.40	18.0

E. coli QC772 carrying the *sodA–lacZ* fusion was transformed with pDT1-5, pSN(−)4, pSNM6, or Sk(−) (the vector in pSN plasmid). Growth conditions were the same as in Table 2. Samples were taken at different time intervals and assayed for growth (OD_{600}) and β-galactosidase (37). Only data from the midlogarithmic phase of growth (2 hr) are presented for briefness.

We therefore reasoned that, if indeed, the mutation in plasmid pSNM6 has altered the binding site for the repressor protein, then its presence in the *sodA–lacZ* fusion strain would not titrate the repressor and therefore should have no effect on the anaerobic expression of β-galactosidase. On the other hand, pSN(−)4, which has the intact wild-type sequence in the postulated regulatory region, should be able to titrate the repressor and therefore cause the anaerobic induction of β-galactosidase. Indeed, this was the case. Data in Table 3 indicate clearly that under anaerobic conditions the *sodA–lacZ* fusion strain harboring pDT1-5 showed a slight induction of β-galactosidase, whereas the presence of pSN(−)4 caused a dramatic 8- to 9-fold induction of the enzyme. The data also suggest that because of the higher copy number per cell of pSN(−)4 vs. pDT1-5, pSN(−)4 was more efficient in titrating the repressor molecule and caused a greater expression of *sodA–lacZ* gene product. On the other hand, both the mutant plasmid (pSNM6) and its vector [SK(−)] were unable to induce β-galactosidase under anaerobiosis (Table 3).

DISCUSSION

The biosynthesis of SodA in *E. coli* is under rigorous and complex control. The enzyme is induced by conditions known to increase the intracellular flux of superoxide (5, 10–12), to deplete the concentration of iron available to the cells (13, 14), or to cause a positive change in the redox status of the cells (9, 17, 44). The synthesis of SodA is also subject to glucose effect (45) and to modulation by changes in the specific growth rate of the organism (46). Furthermore, the regulation of the *sodA* gene in *E. coli* is independent of the inducible DNA repair system (SOS) (47), the oxidative stress regulon (*oxyR*) (19, 48), and the heat-shock regulon (*htpR*) (19, 49). Moody and Hassan (13) proposed a regulatory model based on a negatively controlled operon, in which a redox-sensitive iron-containing protein is proposed to play the role of a trans-acting repressor. The same observations were explained by another model based on autogenous regulation (14). Support for the first model comes from the following observations: (*i*) cell-free extracts from anaerobically grown cells do not contain SodA antigen (17, 27); (*ii*) active SodA is induced anaerobically by the addition of isopropyl D-thiogalactopyranoside to a strain of *E. coli* containing a

ptac-sodA operon fusion (19); (*iii*) β-galactosidase is induced by paraquat, oxygen, and iron chelators in a strain carrying a *sodA–lacZ* fusion (19); (*iv*) the introduction of a multicopy plasmid harboring *sodA* gene into wild-type *E. coli* (9) or into a *sodA–lacZ* fusion strain (19) causes the expression of SodA or β-galactosidase, respectively; and (*v*) the nucleotide sequence of *sodA* indicates the possible presence of a potential binding site for a regulatory protein at the −35 region (23).

The data presented here show that alterations in nucleotide −48 through −44, which are within and near the 19-bp palindrome in the *sodA* gene sequence, resulted in constitutive expression of active SodA. Thus, the mutated *sodA* gene was not inducible by paraquat or by 2,2′-dipyridyl in aerobiosis (Table 1) or by 2,2′-dipyridyl under anaerobic conditions (Table 2). Furthermore, a multicopy plasmid harboring the mutated *sodA* gene (pSNM6) was unable to titrate the repressor molecules present in a *E. coli* strain carrying the *sodA–lacZ* fusion, in contrast to plasmids [pDT1-5 and pSN(−)4] harboring the wild-type gene (Table 3). These results indicate that the region within and around the mutated sequence probably plays an important role in *sodA* regulation and that the mutation disrupts a site necessary for repressor/operator interactions. Furthermore, the aerobic expression of active SodA by cells harboring the mutant plasmid (pSNM6) was 4.6 times higher than that obtained anaerobically (Tables 1 and 2). This could be due to the presence of other regulatory elements or due to posttranslational control. Indeed, a recent report (19) suggests that *sodA* may also be under positive transcriptional control by superoxide radical. Thus, results reported herein do not rule out the possibility that sequences besides the ones we changed could contribute to the regulation of *sodA*.

It is interesting to note that the mutated region overlaps an IHF binding site (i.e., nucleotides −51 through −39) (23, 25). The physiological role of IHF in *E. coli* is not clear, but it has been shown to modulate the expression of several bacterial and viral genes (26, 50). Further studies into the role of IHF in the regulation of SodA biosynthesis are under way and should greatly add to our understanding of controls over *sodA*.

The product of the *fur* (ferric iron uptake regulation) gene, Fur, negatively controls the transcription of many genes required for the synthesis of iron siderophores in *E. coli* (51, 52). Since the synthesis of SodA is derepressed by iron limitation (13, 14, 19), we compared the 5′ region of *sodA* with the sequences of Fur-binding sites (53). A strong Fur-binding consensus sequence (iron-box) was identified within the 19-bp palindrome (see Fig. 2) of *sodA* gene. However, we found that the expression of *sodA* is not affected by mutations in the *fur* gene and that SodA is equally induced in *fur*$^-$ and *fur*$^+$ strains of *E. coli* by the addition of paraquat or 2,2′-dipyridyl to the growth medium (J. R. Schiavone and H.M.H., unpublished results). Similar findings have been reported, and it was concluded that the regulation of *sodA* is independent of the *fur* regulon (54). In conclusion, the present findings corroborate our proposed model for the regulation of *sodA* gene (9, 13) by showing the existence of a cis-acting regulatory site at the −35 region of *sodA*.

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1. McCord, J. M. & Fridovich, I. (1969) *J. Biol. Chem.* **244,** 6049–6055.
2. Hassan, H. M. & Fridovich, I. (1980) in *Enzymatic Basis of Detoxification*, ed. Jakoby, W. B. (Academic, New York), Vol. 1, pp. 311–332.
3. Fridovich, I. (1986) *Adv. Enzymol.* **58,** 62–97.
4. Hassan, H. M. (1989) *Adv. Genet.* **26,** 65–97.
5. Hassan, H. M. & Fridovich, I. (1977) *J. Bacteriol.* **129,** 1574–1583.
6. Keele, B. B., Jr., McCord, J. M. & Fridovich, I. (1970) *J. Biol. Chem.* **245,** 6176–6181.
7. Yost, F. J., Jr., & Fridovich, I. (1973) *J. Biol. Chem.* **248,** 4905–4908.
8. Dougherty, H. W., Sadowski, S. J. & Baker, E. E. (1978) *J. Biol. Chem.* **253,** 5220–5223.
9. Hassan, H. M. & Moody, C. S. (1987) *J. Biol. Chem.* **262,** 17173–17177.
10. Gregory, E. M., Yost, F. J., Jr., & Fridovich, I. (1973) *J. Bacteriol.* **115,** 987–991.
11. Hassan, H. M. & Fridovich, I. (1977) *J. Biol. Chem.* **252,** 7667–7672.
12. Hassan, H. M. & Fridovich, I. (1979) *Arch. Biochem. Biophys.* **196,** 385–395.
13. Moody, C. S. & Hassan, H. M. (1984) *J. Biol. Chem.* **259,** 12821–12825.
14. Pugh, S. Y. R. & Fridovich, I. (1985) *J. Bacteriol.* **162,** 196–202.
15. Miyake, K. (1986) *J. Gen. Appl. Microbiol.* **32,** 527–533.
16. Smith, M. W. & Neidhardt, F. C. (1983) *J. Bacteriol.* **154,** 344–350.
17. Schiavone, J. R. & Hassan, H. M. (1988) *J. Biol. Chem.* **263,** 4269–4273.
18. Privalle, C. T. & Fridovich, I. (1988) *J. Biol. Chem.* **263,** 4274–4279.
19. Touati, D. (1988) *J. Bacteriol.* **170,** 2511–2520.
20. Gardner, P. R. & Fridovich, I. (1987) *J. Biol. Chem.* **262,** 17591–17595.
21. Gardner, P. R. & Fridovich, I. (1988) in *Oxyradicals in Molecular Biology and Pathology*, UCLA Symposia on Molecular and Cellular Biology, eds. Cerutti, P. A., Fridovich, I. & McCord, J. M. (Liss, New York), Vol. 82, pp. 163–172.
22. Touati, D. (1983) *J. Bacteriol.* **155,** 1078–1087.
23. Takeda, Y. & Avila, H. (1986) *Nucleic Acids Res.* **14,** 4577–4589.
24. deCrombrugghe, B., Busby, S. & Buc, H. (1984) *Science* **224,** 831–838.
25. Craig, N. L. & Nash, H. A. (1984) *Cell* **39,** 707–716.
26. Friden, P., Voelkel, K., Sternglanz, R. & Freundlich, M. (1984) *J. Mol. Biol.* **172,** 573–579.
27. Carlioz, A. & Touati, D. (1986) *EMBO J.* **5,** 623–630.
28. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
29. Birnboim, H. C. & Doly, J. (1979) *Nucleic Acids Res.* **7,** 1513–1523.
30. Zoller, M. J. & Smith, M. (1983) *Methods Enzymol.* **100,** 468–500.
31. Grunstein, M. & Hogness, D. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 3961–3965.
32. Benton, W. & Davis, R. (1977) *Science* **196,** 180–182.
33. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
34. Beauchamp, C. & Fridovich, I. (1971) *Anal. Biochem.* **44,** 276–287.
35. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121,** 404–427.
36. Cohenford, M. A., Abraham, J. & Medeiros, A. N. (1988) *Anal. Biochem.* **168,** 252–258.
37. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab., Cold Spring Harbor, New York), pp. 352–355.
38. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
39. Towbin, H., Stachelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
40. Gregory, E. M. & Fridovich, I. (1974) *Anal. Biochem.* **58,** 57–62.
41. Archibald, F. S. & Fridovich, I. (1981) *J. Bacteriol.* **146,** 928–936.
42. Uhlin, B. E. & Nordstrom, K. (1977) *Plasmid* **1,** 1–7.
43. Minton, N. P., Chambers, S. P., Prior, S. E., Cole, S. T. & Garnier, T. (1988) *BRL Focus* **10,** 56.
44. Whiteside, C. & Hassan, H. M. (1987) *Arch. Biochem. Biophys.* **257,** 464–471.
45. Hassan, H. M. & Fridovich, I. (1977) *J. Bacteriol.* **132,** 505–510.
46. Hassan, H. M. & Fridovich, I. (1977) *J. Bacteriol.* **130,** 805–811.
47. Hancock, L. C. & Hassan, H. M. (1985) *J. Biol. Chem.* **260,** 12954–12956.
48. Bowen, S. W. & Hassan, H. M. (1988) *J. Biol. Chem.* **263,** 14808–14811.
49. Hassan, H. M. & Lee, F.-J. S. (1989) *FEMS Microbiol. Lett.* **58,** 133–138.
50. Friedman, D. I., Olson, E. R., Carver, D. & Gellert, M. (1984) *J. Bacteriol.* **157,** 484–489.
51. Hantke, K. (1981) *Mol. Gen. Genet.* **182,** 282–292.
52. Bagg, A. & Neilands, J. B. (1985) *J. Bacteriol.* **161,** 450–453.
53. Calderwood, S. B. & Mekalanos, J. J. (1987) *J. Bacteriol.* **169,** 4759–4764.
54. Niederhoffer, E. C., Naranjo, C. M. & Fee, J. A. (1989) in *Metal Ion Homeostasis: Molecular Biology and Chemistry* (Liss, New York), pp. 149–158.